A STUDY OF THE INTERACTIONS OF β-CARBOLINE ALKALOIDS WITH DNA OLIGOMERS BY VARIOUS ANALYTICAL METHODS

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By

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INTRODUCTION

Cancer is one of the most common shocking disease affecting about 6 millions of people per year. Cancer is the most common name given by the ancient Romans for a large group of diseases that are characterized by three basic features:

1. Uncontrolled cell proliferation;
2. Loss of cellular differentiation; and
3. The ability to invade surrounding tissues and to establish new growth thereafter.

There are a number of other terms to describe cancer, which are either more descriptive or more specific e.g.,“ tumor ”, “neoplasm”, and the root “onco” all relate to a swelling of tissue. Cancer is the second most common cause of death[1] in the developed world and a similar trend has emerged in the developing countries too. Cancer occurrence[2] in India is estimated to be around 2.5 million, with over 8, 00,000 new cases and 5, 50,000 deaths occurring each year due to this disease.

There are many ways to treat cancer medicinally. Surgery, radiotherapy, chemotherapy and immunotherapy are the most common treatments to choose. Among these cancer treatment methods, chemotherapy is a relatively new one. Systematic chemotherapy only made its appearance in the middle of World War II, when Farber prescribed methotrexate to treat childhood leukemia in 1940. Since then enormous progress in the drug design, methods of delivering the drugs, and to reduce the toxic side effects has been made, especially in recent decades. Chemotherapeutic agents work by interfering with the process by which cancer cells divide to produce new cells. The drugs are introduced into the bloodstream and circulate around the body, killing cancer cells that reside at the original site of occurrence as well as those migrated to other tissues (metastasis). This has a great advantage over other methods in which treatments can only be applied locally to a particular part of the body. Chemotherapeutic agents are of both natural and synthetic origin.

In the course of searching for new, more effective anticancer agents, natural products become an extremely important, productive and readily available domain. About 30 plant derived compounds have been isolated so far and are currently under clinical trials. Many anti-cancer agents have been isolated from various plant sources like *Catharanthus roseus*, *Podophyllum* species, *Taxus brevifolia*, *Camptotheca acuminate*, *Betula alba*, *Cephalotaxus* species, *Erythroxylum pervillei*, *Curcuma longa*, *Ipomoea batatas*, *Centaurea schischkinii* and many others. Scientists are still attempting to explore the bioavailability of anti-cancerous compounds in unexplored plant species.

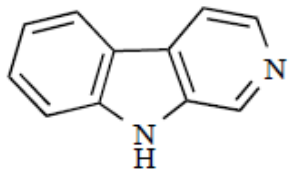
There are four major structural classifications of plant-derived anticancerous compounds *viz*., Vinca alkaloids, Epipodophyllotoxin lignans, Taxane diterpenoids and Camptothecin quinoline alkaloid derivatives.Vinca alkaloids belong to an important class of anticancer drugs. These drugs are obtained from plant Catharanthus roseus. The most active compounds with anticancer activity, belonging to this class are Vinblastine and Vincristine. These compounds have exhibited potential activity against lymphocytic leukemia.

Except Vinca alkaloids there are many others alkaloids e.g. Etopside, Teniposide, Taxol, Camptothecin, Topotecan, Berberine, Colchicines, Cucurbitacin, Ellipticine, Emodin, Flavopiridol, Palmitine, Silvestrol are promising anticancer agents. The plant-derived anticancer drugs or the plant derived cancer chemotherapeutic agents were responsible for approximately one third of the total anticancer drug sales worldwide, or just under $4 billion dollars in 2007; namely, the Taxanes, Paclitaxel and Docetaxel, and the Camptothecin derivatives, Irinotecan, Topotecan, etc.

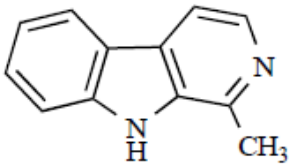
β-CARBOLINE ALKALOIDS

β–Carboline alkaloids[3,4] are a large group of natural and synthetic indole alkaloids that possess a common tricyclic pyrido [3,4-b] indole ring structure. They are found[5-7] in alcoholic beverages, tobacco smoke and animal fluids and tissues, sometimes with an endogenous origin. These molecules can be categorized according to the saturation of their N-containing, six-membered ring. Unsaturated members are named as fully aromatic β-carbolines [Norharman (**1**), Harman (**2**), Harmine (**3**), Harmol (**4**)] whereas the partially or completely saturated ones are known as dihydro-β-carbolines [Harmaline (**5**), Harmalol (**6**)] and tetrahydro-β-carbolines (THBCs, **7**), respectively (Figure 1). Among these compounds, 3-substituted and 9-substituted β-carboline derivatives have also been synthesized and these derivatives like their parent molecule showed

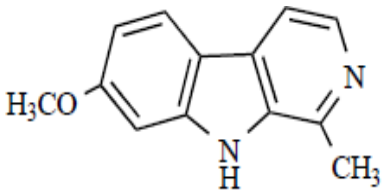
notable pharmacological activities. Those tricyclic compounds usually contain several substituents both in the pyrido ring and/or the indole ring.



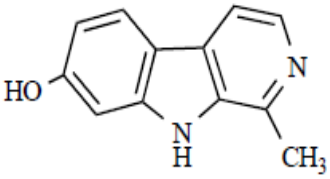
Norharman (1)



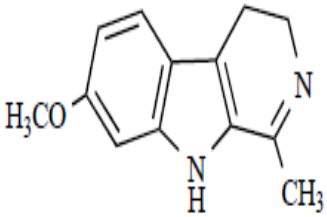
Harman (2)



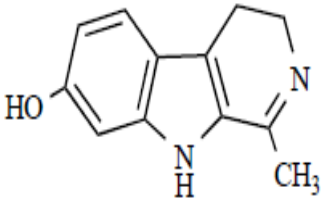
Harmine (3)



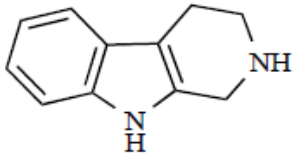
Harmol (4)



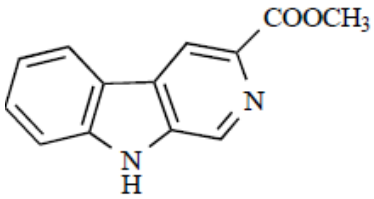
Harmaline (5)



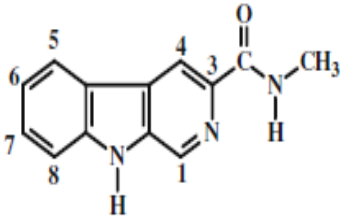
Harmalol (6)



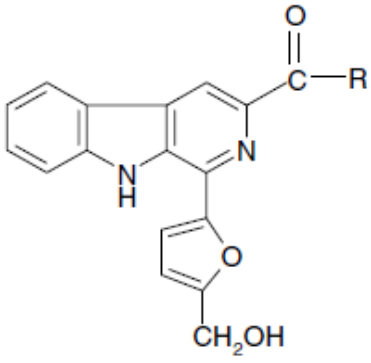
THBC (7)



β-CCM (8)



β-CMAM (9)



Flazinamide (11)

Figure 1: β–Carboline alkaloids

The β-carboline alkaloids were originally isolated from *Peganum harmala* (Syrian Rue, family Zygophillaceae), which is being used as a traditional herbal drug[8] as an emmenagogue and abortifacient in the Middle East and North Africa. Plants containing β-carbolines were widely used as hallucinogenic drinks or snuffs in the Amazon basin. During the last two decades, numerous simple and complicated β-carboline alkaloids with saturated or unsaturated tricyclic ring system have been isolated and identified from various terrestrial plants as the major bioactive constituents.

β-Carbolines have interesting pharmacological[9] and medical[10] properties and have been used as antiviral,[11] antimicrobial,[11] hallucinogenic,[12] potential insulin secretagogues,[13] antimalarial[14] or anti-tumour agents.[14-17] These comounds also act as photosensitizers with potential application in photodynamic therapy[18] or as antioxidants,[7,19] although the mutagenic and co-mutagenic properties[20–28] of this class of compounds has been also indicated. They also possess[29] anti-HIV and antiparasitic activities. β-Carbolines exhibited[30] cytotoxicity with regards to HL60 and K562 leukemia cell lines. These compounds have been reported to possess significant antitumor activities. Ground *P. harmala* seeds have been used[31] occasionally to treat skin cancer and subcutaneous cancers traditionally in Morraco.

In Iran and China, the extracts containing β-carbolines from the plant *P. harmala* have been widely used[32, 33] as a very potent antitumor folk medicine for cancers of digestive system. Since β-carbolines are of pharmacological importance, particularly in cancer treatment, much attention and numerous interdisciplinary studies have been focused on their biological effects.

DRUG-DNA INTERACTIONS

The biological effects of the drugs are strictly connected to their ability to interact with nucleic acids and proteins. The physical and molecular basis of natural drugs with nucleic acid structures have been a subject of extensive study[34-36] in the recent past years. The mode of action of many natural drugs in current chemical use for the treatment of cancer, genetic disorders, and microbial and viral diseases is believed to be based on their highly specific but non-covalent and reversible intercalative binding to the genetic material.[37,38] Virtually all of the current clinical and experimental antitumor drugs are thought to act by disruption[39] of nucleic acid metabolism at some level. The class of antitumor compounds that has received most development recently is the DNA binding agents that bind tightly, but reversibly to DNA by a combination of hydrophobic, electrostatic, H-bonding, and dipolar forces.

There are two principal modes for non-covalent binding to DNA (a) intercalation and (b) minor groove binding.[40-43] Intercalating drugs have planar, polyaromatic ring systems in which drug inserts between two adjacent base pairs in a DNA double helix. The drug-DNA complex is stabilized by π–π hydrophobic and van der Waal's interactions between the DNA bases and the drug molecule. The examples of intercalator drugs are ethidium bromide, Daunomycin, actinomycin etc. (Figure 2)

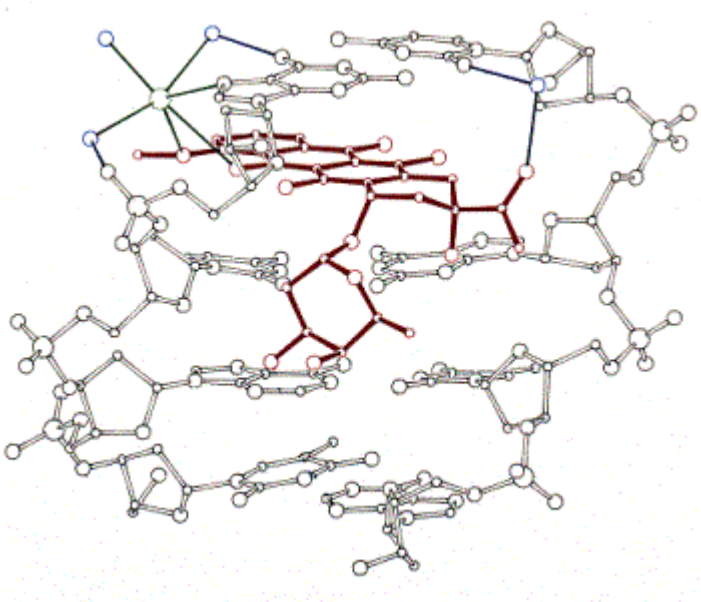


Figure 2: *the crystal structure of a daunomycin intercalated into the minor groove of DNA.*

The second mode of drug binding to DNA is the minor groove binding. These drugs consist of several aromatic rings viz. pyrrole, immidazol, indole etc. These rings are connected by amide bonds which have torsional freedom. Such ligands typically have a crescent-shape which allows sterically favorable fit in the minor groove of DNA and the drug aligns[44-46] itself on the binding site in the DNA minor groove. Examples of minor groove binders are Hoechst 33258, Netropsin, Distamycin etc. (Figure 3)

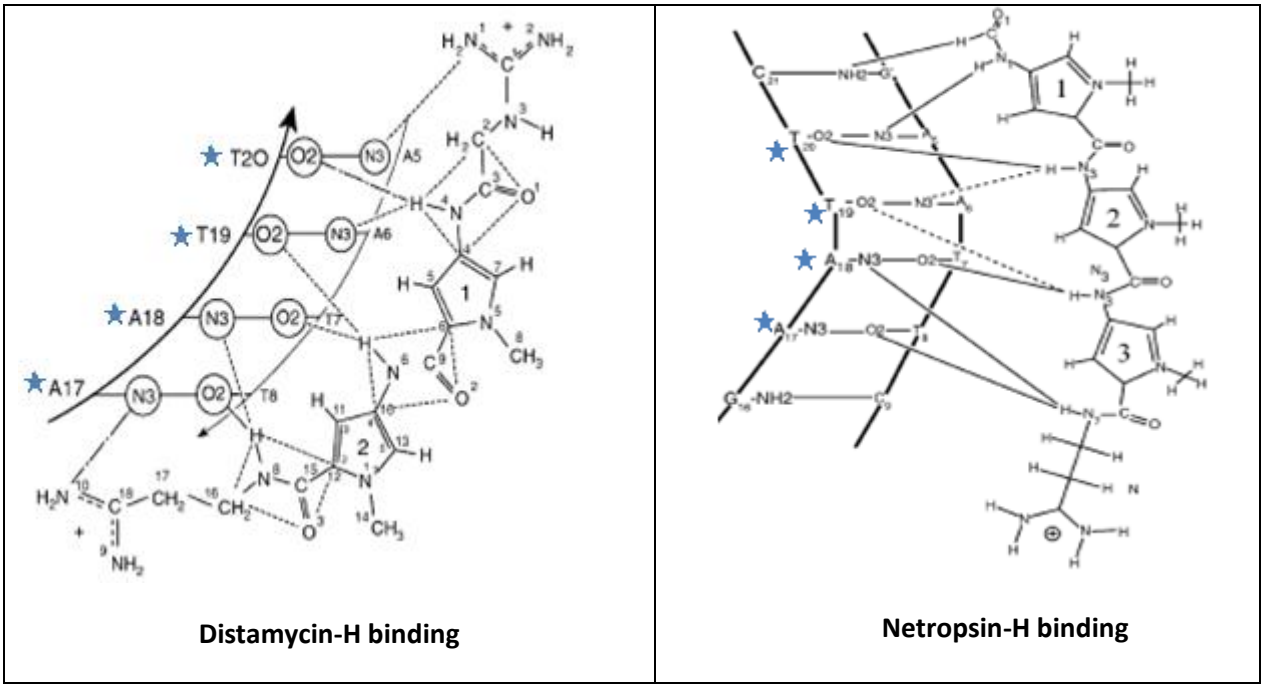


Figure 3: Hydrogen-bonding scheme of distamycin and netropsin with dodecamer duplex.

Many aspects of the biological activity[47,48] of β-carbolines are related to their interaction with DNA, which is reported to occur by intercalation. The planar β-carboline ring is suggested to be sandwiched between adjacent base pairs of double stranded DNA (dsDNA), stabilized[47,48] by extensive van der Waals and H-bonding interactions along the groove of the DNA helix. The drug-DNA interactions showed[49] high selectivity towards the G-C base pairs.

The determination of the binding constant of a drug for its target molecule is of considerable importance. It is a basic experimental parameter in a variety of studies, such as the prediction of drug efficiency, or in the pharmacokinetic drug interaction. The interactions of β-carboline alkaloids with DNA have been investigated by different methods. All of the results[50-56] showed that these compounds intercalated into DNA with different degrees.

CURRENT STATUS OF THE STUDY

Cao *et al.* reported[17] that Harmine and its 9-substituted derivatives exhibited remarkable DNA intercalation activities and the potency of intercalation into DNA were enhanced significantly by introducing an appropriate substituent into position-9 of β-carboline nucleus. Xiao[47] et al. synthesized a series of 3-substituted-β-carboline derivatives from l-tryptophan. The intercalating binding mode of these compounds with DNA, the effects of the flexible alkylamine side chain on the intercalating ability and their antitumor activity were studied, which agreed well with the molecular modeling results.

Tamura[48] *et al.* studied affinity of 3, 4, β-carboline, and 3-amino-β-carboline to a DNA oligomer or calf thymus DNA spectrophotometrically. Optical spectroscopy of these β-carbolines showed 25 ~ 65 nm bathochromic shifts of the light absorption bands upon binding to the nucleic acids, which suggests binding by intercalation.

Feigon[49] *et al.* used 1D ^{1}H-NMR to study the interaction of 70 antitumor drugs (including Norharman, Methylnorharman, Methylharman and Methylharmine) with DNA. Spectra of the low-field (H-bonded imino proton) resonances of DNA were studied as a function of drug per base pair ratio. This study showed that all the 4 β-carboline alkaloids were G-C base pair specific intercalaors.

Tiara[50] *et al.* studied the interaction of six β-carboline derivatives Norharman, Harman, Harmol, Harmine, Harmalol and Harmaline with superhelical plasmid pBR322 DNA by absorption spectroscopy, ethidium bromide displacement method, circular dichroism and fluorescence techniques. The results showed intercalation of all these drugs into DNA. The magnitude of intercalation except for Harmalol decreased in the order of:

Harmol > Harmine > Harmaline > Harman > Norharman

Hayashi[51] *et al.* found that Harman and Norharman reacted with DNA by intercalation, resulting to a quenching of the fluorescence of Norharman and a marked red shift and hypochromism of the absorption spectra of Norharman and Harman. However Harman intercalated more easily into DNA than Norharman.

Duportail[52] *et al.* studied the binding to calf thymus DNA of the hallucinogen Harmine and one of its analogues Harmaline by absorption spectrophotometry and fluorescence quenching analysis. Viscosity measurements were also carried out. For both molecules, quenched and

unquenched sites on DNA are present. For each type of binding site, the value of the product of the number of sites times the association constant was determined. Harmine is more strongly bound than Harmaline. Viscosity measurements indicate intercalation in the case of Harmine only.

Duportail[55] studied the interaction of the β-carboline derivatives Harmine and Harmaline with calf thymus DNA using linear and circular dichroism techniques. The 82° angle found for Harmine is compatible with the interpretation that the molecule is intercalated between the two consecutive base pairs of the DNA. Since a 67° angle was found for Harmaline, an intercalation is in that case unlikely; the molecule is probably in a tilted position in one of the DNA grooves. This study showed that the hydrogenation of a double bond of the pyridine ring converting Harmine to Harmaline greatly altered the interactions of the molecule with DNA.

Zubiri[57] *et al.* recently showed that β-carboline-3-carboxylic acid N-methylamide (β-CMAM) also interacts with single stranded DNA, ssDNA, and with the polynucleotides: polyadenylic (Poly A), polycytidylic (Poly C), polyguanylic (Poly G), polythymidylic (Poly T) and polyuridylic (Poly U) acids. The interaction is stronger with ds-DNA than with ss-DNA, and showed selectivity towards Poly G, suggesting that intercalation may involve some specific interactions, such as with guanine in the G-C base pair. However, the fact that β-CMAM also interacts with ss-DNA and oligonucleotides showed that intercalation is not the only mechanism involved in β-CMAM-polynucleotide interactions.

Nafisi[58] *et al.* reported that β-carboline alkaloids also interact with RNA. They investigated the binding of β-carboline (Harmine, Harmane, Harmaline, Harmalol, and Tryptoline) with yeast RNA. Fourier transform infrared (FT-IR) and UV-visible spectroscopic methods were used to determine the ligand-binding modes, the binding constants, and the stability of alkaloid-RNA complexes in aqueous solution. Spectroscopic evidence showed major binding of alkaloids to RNA. The secondary structural changes in biopolymer were not observed upon alkaloid interaction and RNA remains in the A-family structure in these complexes.

Wang[59] *et al.* synthesized Flazinamide [1-(5'- hydromethyl-2'-furyl)-β-carboline-3-carboxamide] which has a similar structure as Flazin (weak anti-HIV drug). Flazinamide also showed anti-HIV properties. The cytotoxicity of Flazinamide was about 4.1 fold lower than that of Flazin. The conversion of the carboxyl group in 3 position of Flazin markedly enhanced the anti-viral activity and Flazinamide might interfere in the early stage of HIV life cycle.

Iñigo[60] *et al.* studied interaction between β-Carboline-3-carboxylic acid N-methylamide (β-CMAM) and nucleobases, nucleosides and nucleotides in the ground state with UV-visible, ^{1}H-NMR and ^{31}P-NMR spectroscopies and in the first excited state, with steady-state and time-resolved fluorescence spectroscopy. Job plots showed a predominant 1:1 interaction in both electronic states.

Du[61] *et al.* studied the interactions between Harmine and oligonucleotides $d(GTGCAC)_2$. The binding thermodynamic parameters were obtained by Isothermal Thermal Cooling (ITC) method. Harmine binds to oligonuleotide duplex $d(GTGCAC)_2$ in a fashion of intercalation mode and specific interaction and nonspecific interactions existed in the binding process. The ^{1}H-NMR experiments indicated that oligonucleotides $d(GTGCAC)_2$ was a generally self-complementary B-type duplex molecule, and harmine bound to DNA duplex $d(GTGCAC)_2$ at the side of methoxyl group preferentially in a mode of intercalation.

FORMULATION OF THE PROBLEM

It has been reported that β-carboline alkaloids are anticancer agents and also interact with genetic materials like DNA and RNA. A detailed structural work is needed to be furnished on the interactions of β-carboline alkaloids with DNA. These studies may serve as a standard reference as the objective of our research. Different techniques have been used for these studies but the detailed structure of these drugs and DNA complex has not been studied as yet. Little is known about the orientation of specific bases as well. So we propose techniques like spectrophotometric methods, electrochemical methods, NMR and molecular modeling for the evaluation of the drug-DNA interactons of Norhaman and derivatives. These techniques will provide us the structural information about the drug-DNA complexes and their interactions with the nucleic acids inside the cell.

OBJECTIVES

Main objectives of the proposed study are:

1. To study the interaction of the proposed β-carbolines with 4 DNA oligomeric sequences by Fluorescence/UV/CD spectroscopic techniques and calculate the binding constants.
2. To study the interaction of the proposed β-carbolines with 4 DNA oligomeric sequences by electrochemical techniques.
3. To study the detailed structure of a drug-DNA complex by 2D-NMR methods.
4. To study the detailed structure of the drug-DNA complexes by molecular modeling.

MATERIALS AND METHOD

MATERIALS

The DNA sequences have been chosen to evaluate the AT/GC sequence specificity of the drugs. The proposed DNA sequences for our studies are:

1. DNA-1: 5'-d(GATGGCCATC)$_2$
2. DNA-2: 5'-d(GATCCGGATC)$_2$
3. DNA-3: 5'-d(GGCAATTGCC)$_2$
4. DNA-4: 5'-d(GGCTTAAGCC)$_2$

The following compounds have been proposed for the study since they are β-carboline alkaloids and showed anticancer properties.

1. Norharman **(1)**
2. Harman **(2)**
3. Harmine **(3)**
4. Harmaline **(4)**
5. Harmol **(5)**
6. Harmalol **(6)**

METHODOLOGY

SAMPLE PREPARATION

As the first step, the DNA solution will be prepared in the phosphate buffer at physiological pH of ~7.4. The concentration of DNA oligomers would be determined spectrophotometrically using the molar extinction coefficients. The concentration of alkaloids would be calculated volumetrically. All experiments will be carried out at the physiological pH.

FLUORESCENCE STUDIES

Equilibrium Binding Titration technique has found universal application[62] in the drug-DNA binding studies. Essentially, an alkaloid solution of fixed concentration is transferred to a thermostated cuvet and the progressive absorbance or fluorescence changes are recorded after an addition of serial aliquot of a DNA solution. Optical changes are normally analyzed for the drug component in terms of free drug and the bound drug in the resulting complex. For the binding study of the alkaloid-DNA complexes, the titration would be carried out by using fluorescence spectroscopy, as most of the alkaloids produced fluorescence.

CIRCULAR DICHROISM STUDIES

When a drug binds to DNA, its electronic structure is affected, and it also perturbs the electronic structure of the DNA. The resulting change to the electronic spectroscopy can be used to probe the drug-DNA interactions. Circular dichroism has been effectively used in interpreting the structural nature of DNA upon drug binding. Circular dichroism spectroscopy involves measuring the difference in absorption of left and right circularly polarized light. It is uniquely sensitive to the helicity of the molecules being studied.

ELECTROCHEMICAL STUDIES

The electrochemical analysis of the drug-DNA interactions is mainly based on the differences in the redox behavior of the nucleic acid-binding molecules in the absence and presence of DNA, including the shifts of the formal potential of the redox couple and the decrease of the peak current resulting from the dramatic drop in the diffusion coefficient after association with DNA.[63]

For DNA biosensor fabrication, DNA will be immobilized over the working surface of glassy carbon electrode. Prior to immobilization, glassy carbon electrode (GCE) is polished by using an alumina cleaning kit until a mirror like surface is obtained. Then the electrode is sonicated to remove alumina from the surface of the electrode, thoroughly washed by using deionized water,

and dried. After this, DNA of specific concentrations immobilized on the GCE surface, and then allowed to dry for 30 min at room temperature. After this the DNA biosensor is placed in drug solution and the differential pulse voltammogram signals are recorded. The optimization of the experimental conditions is furnished for the buffered solution (phosphate buffer, BR buffer and acetate buffer) at varying pH, for surfactants (cationic, anionic and non-ionic), at varying scan rate and for different concentrations of the drugs**.**

NMR STUDIES

As the DNA binding profiles of these alkaloids are evaluated, it would be pertinent to study at least one of the complexes by more precise technique[64] i.e. NMR to obtain the elaborated molecular structure. 1D and 2D ^{1}H-NMR experiments would be conducted on DNA oligomers as reported.[65,66] Multiple 2D ^{1}H-NOESY experiments (at different mixing time intervals) would be furnished in order to obtain the detailed spectra for extracting the spatial proximity information about various hydrogen atoms of alkaloids and DNA oligomers.

MOLECULAR MODELING STUDIES

After obtaining the structural details of the alkaloid-DNA complex, Molecular Modeling and Molecular Dynamics experiments may be carried out by using XPLOR software in order to furnish an NMR refined structure of the DNA duplex and the drug-DNA complex.

FUTURE SCOPE OF THE STUDY

The problem of cancer has reached panic proportions despite the ongoing scientific developments to fight this disease. We need to further enrich the knowledge about how anticancer agents work so that we can use these drugs more effectively against this disease. This knowledge in turn can further be used in the management of various other diseases where DNA plays a significant role. Understanding the exact mechanism will help in developing and designing new compounds, which target DNA in one way or the other more effectively. Keeping this in mind, our objective is to obtain structural information regarding the drug-DNA interactions. This comprehensive study may lead to the generation of data base of structural information, which would be extremely helpful to design anticancer agents with improved and better biological activity.

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